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13. ABSTRACT (Maximum 200 Words)

Estrogen can be metabolized to hydroxylated catechol estrogen, a genotoxic metabolite of estrogen, which causes DNA damage and tumors in animal models. In situ synthesis of estrogen in the breast through aromatase results in high tissue estrogen concentrations. We hypothesized that overexpression of aromatase in breast tissue increases tissue estradiol concentrations and consequent genotoxic metabolites, and eventually causes breast cancer. To test our hypothesis, we stably expressed aromatase cDNA in MCF-10A cells, a benign breast epithelial cell line (MCF-10A^{arom}). We demonstrated that MCF-10A^{arom} cells expressed functional aromatase using tritiated water release assay and products isolation by thin layer chromatography. MCF-10A^{arom} cells, incubated for 3 months with aromatase substrate, androstenedione, formed colonies in soft agar indicating the overexpression of aromatase induces cellular transformation. MCF-10A^{arom} cells have all enzymes required to convert estrogen to catechoestrogens and quinine. Overexpression of aromatase enhanced production of genotoxic metabolites, which could be blocked by aromatase inhibitor, letrozole. MCF-10A^{arom} cells did not form palpable tumor in nude mice suggesting that multiple factors are required for breast cancer initiation.

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Introduction

Clinical data and results from animal studies indicate that estrogen not only promotes breast cancer growth but also plays a role in breast cancer initiation. The carcinogenic effect of estrogen is mediated by hydroxylated catechol estrogen, a genotoxic metabolite of estrogen, which causes DNA damage (1-4). In situ synthesis of estrogen in the breast through aromatase makes the major contribution to the high tissue estrogen concentrations (5). We hypothesized that overexpression of aromatase in breast tissue increases tissue estradiol concentrations and consequent genotoxic metabolites, and eventually causes breast cancer. To test our hypothesis, we have established a stable cell line of benign breast epithelial cell by expressing aromatase gene in MCF-10A cells. Using this model, we investigated the synthesis and metabolism of estrogen, formation of depurinated DNA adduct and their potential in cellular transformation and tumorigenesis.

Body

I. Specific aim 1: Establish an in vitro model of aromatase overexpression by stable transfection of human aromatase cDNA into a benign breast epithelial cell line, MCF-10A. We realize that to study the role of aromatase in breast cancer development, a model allowing quantitative study of the relationship between aromatase activity and biological effects of estrogen is required. The Tet-Off expression system allows regulated expression of aromatase by adjusting tetracycline concentrations. Using this technique, MCF-10A cells will be double-transfected with a regulatory vector (pTet-Off) and the aromatase expressing vector (pTRE-arom).

We initially plan to express aromatase in MCF-10A cells under the control of tetracycline so that we can quantitatively study the relationship between aromatase activity and its biological effect in breast cancer initiation. We chose to use Tet-Off gene expression system from the CLONETECH Company. After careful screening of 45 clones that expressed tetracycline controlled transactivator (tTA), we found that MCF-10A cells are not suitable for tetracycline-controlled gene expression.

As a backup approach, we generated a stable aromatase expressing line of MCF-10A using pHβ-aro vector, a vector being successfully used to generate stable aromatase expressing cells in several different cell lines (6). We screened 66 G418 resistant clones and 23 clones showed aromatase activities as measured by radiometric method using [1β-^{3}H]androstenedione as a substrate. The aromatase activities in selected clones were correlated with cell number, incubation time, and can be completely inhibited by letrozole, a specific aromatase inhibitor. In one clone, the apparent K_m and V_{max} were 9.4 nM and 15.2 pmol/mg/h, respectively. Aromatase activity in transfected MCF-10A cells was also confirmed by formation of radiolabeled estrone and estradiol after incubation of the cells with [1,2,6,7-^{3}H]testosterone. At the end of the first reporting period, we successfully established the model system.

II. Specific aim 2: Determine the presence of key metabolic transformations that lead to DNA damage in aromatase transfected MCF-10A breast epithelial cells (MCF-10Aarom). Estrogen metabolites (2- and 4-

hydroxylated estrogens), catechol estrogen quinone bound to glutathione and DNA will be measured in cell cultures.

MCF-10A^{arom} and MCF-10A^{vect} cells were cultured in 150 mm dishes until confluence. The cells were treated in serum free medium with vehicle, estradiol, 4-OHE_2, androstenedione plus or minus letrozole for 24 h. All compounds were at the concentration of 10 μM. Cells and media were collected and kept at -80C until analysis. Estrogen metabolites, conjugates, and depurinated DNA adducts were measured by HPLC with a 12-channel electrochemical detector and confirmed by mass spectrometry (7-9). Figure 1 illustrates the metabolic pathways of estrogen and measurable metabolites/DNA adducts.

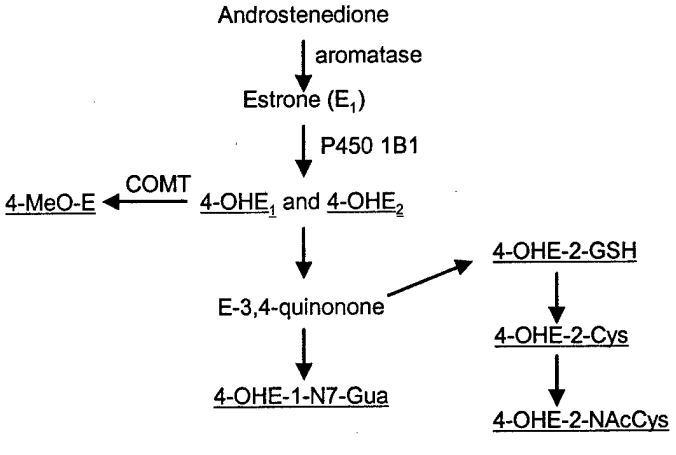

Fig. 1. Synthesis and metabolism of estrogen. Underlined are measurable metabolites.

MCF-10A cells have all enzymes that catalyze conversion of estrogen to catechoestrogens and their methylation (Fig. 2). Incubation of MCF-10A^{arom} cells with estradiol (10 μM) for 24 hours dramatically increased production of 4-hydroxyestrone (Fig. 2A). Incubation with aromatase substrate, androstenedione (Δ^4A), also increased the level of 4-hydroxyestrone (Fig. 2A) indicating that Δ^4A was aromatized to estrogen which was metabolized to 4-hydroxyestrogen. A large portion of catechoestrogens were converted to methoxyestrogens, detoxification metabolites, by catecho-*o*-methyl transferase (COMT). This is more significant when 4-OHE_2 was used as a precursor (Fig. 2B). Estrogen-3,4-quinones (E-,4-Q) are the core genotoxic metabolites of estrogens. Estrogen-3,4-quinones are highly reactive and form conjugates with glutathione or bind to purine motifs on DNA as soon as they form (Fig. 1). Formation of glutathione conjugates and their metabolites reflects production of E-3,4-Q. As shown in Fig. 2C, 4-OHE_2-2-NAcCys, a conjugate of E-3,4-quinone, was detected in MCF-10Aarom cells pretreated with vehicle, E_2, 4-OHE_2, and Δ^4A. Depurinated DNA adduct, 4-OHE_2-1-N7Gua, was only detected in the cells treated with E_2, 4-OHE_2, and Δ^4A. The level of 4-OHE_2-1-N7Gua was undetectable in the cells incubated with Δ^4A plus the specific aromatase inhibitor, letrozole (Fig. 2D).

III. Specific aim 3: Determine the role of aromatase in breast carcinogenesis. Anchorage-independent growth of MCF-10Aarom cells and loss of heterozygosity (LOH) will be examined to assess cell transformation. MCF-10Aarom cells will be inoculated into mammary fat pads of ovariectomized nude mice. The mice will be supplemented with the aromatase substrate, testosterone, plus or minus an aromatase inhibitor. Tumor formation, histological changes and their relation to aromatase expression will be examined.

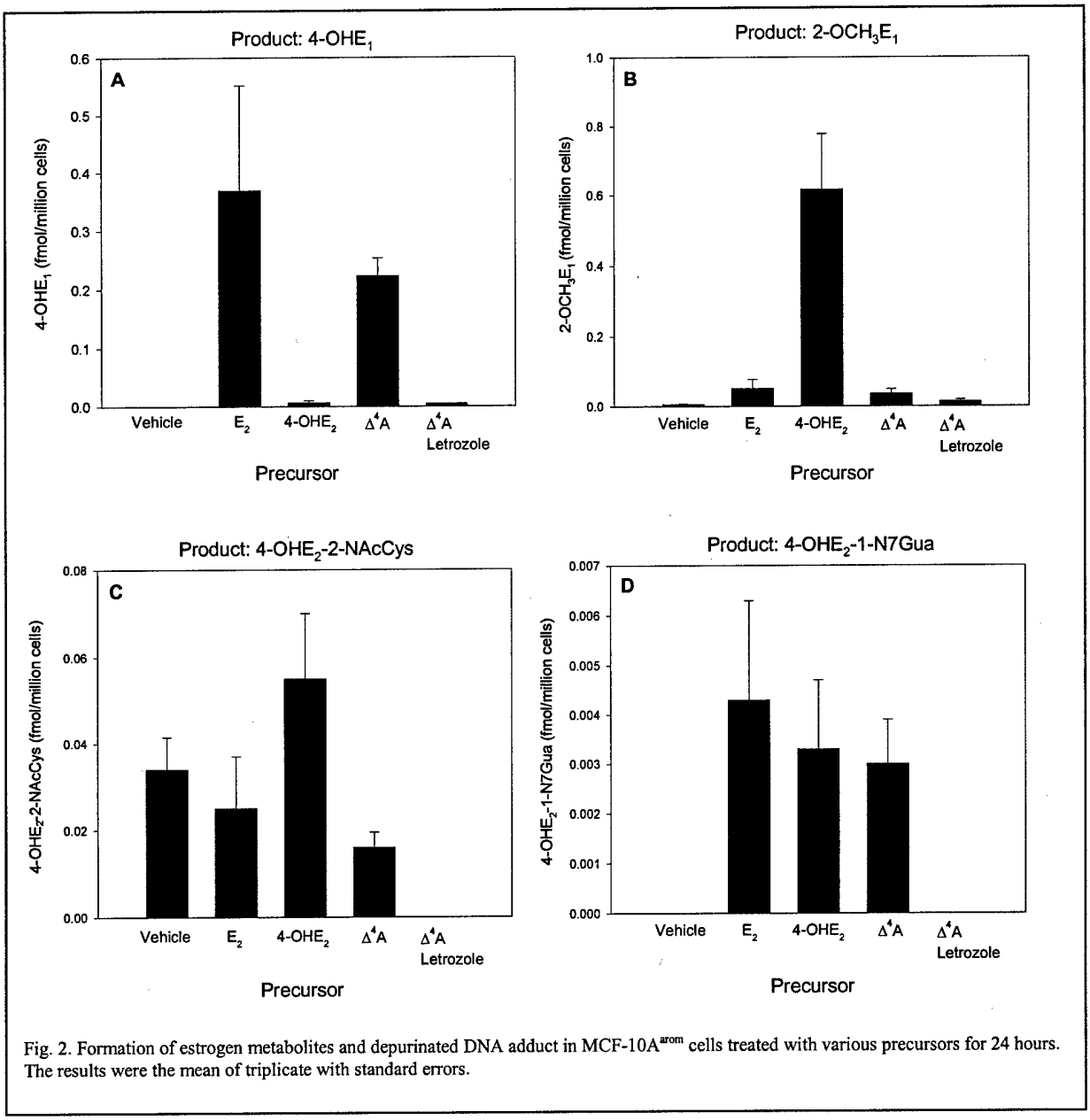

Fig. 2. Formation of estrogen metabolites and depurinated DNA adduct in MCF-10A^{arom} cells treated with various precursors for 24 hours. The results were the mean of triplicate with standard errors.

1. Anchorage independent growth

One characteristics of cellular transformation is anchorage independent growth in soft agar. To determine whether aromatase overexpression results in cellular transformation, the ability of MCF-10A^{arom} cells to grow in soft agar was assessed.

Pretreatment of MCF-10A^{arom} cells with estradiol (10^{-6} M) and androstenedione (10^{-6} M) for 3 months increased the number of colony formed in soft agar. In contrast, regular MCF-10A cells did not grow in soft agar. These data suggest that overexpression of aromatase in benign breast epithelial cells induces cell transformation.

2. Loss of heterozygosity (LOH)

Loss of heterozygosity may contribute to the initiation and progression of breast cancer. LOH has been observed in both in situ and invasive breast carcinomas on multiple chromosomal arms (10, 11) as well as in normal tissue adjacent to breast carcinomas (12). Human breast epithelial cells transformed with estradiol also showed LOH in chromosome 11 (13). To determine whether aromatase overexpression causes gene instability, LOH was assessed in MCF-10A^{arom} cells in collaboration with Dr. Russo at Fox Chase Cancer Center, Philadelphia.

Cell lines: MCF-10A (regular MCF-10A), MCF-10A^{vect} (transfectet with empty pHβ vector) and MCF-10A^{arom} cells.

Treatment: MCF-10A^{arom} cells were treated for 10 months with androstenedione (Δ^4A, 10^{-6} M), Δ^4A + letrozole (10^{-7} M), letrozole alone and estradiol (10^{-6} M). Regular MCF-10A and MCF-10A^{vect} cells were without treatment.

Genomic DNA was extracted LOH was assayed using microsatellite polymorphism makers TP53dint and D13S893 that flank *p53* (chromosome 17) and *WT2* (chromosome 11p15.5) respectively. Both genes are tumor suppress genes.

All samples display one allele of 185 bp for the marker D13S893 and one allele of 295 bp for the marker TP53dint. It is necessary to examine more loci.

3. In vivo study

To determine whether aromatase overexpression causes genotoxic damage and consequently mammary tumor, tumorigenesis of aromatase expressing MCF-10 cells were assessed in nude mice. Long term experiment for tumor formation and prevention was carried out during this report period.

Ovariectomized nude mice were inoculated subcutaneously with 5 million MCF-10A^{arom} on left flank and regular MCF-10A cells on right flank. The animals were then divided into three groups, 20 mice each. One group of animals received androstenedione silastic capsule to provide aromatase precursor. One group of animals received androstenedione plus letrozole (5 μg/day, s.c.). The third group served as a control. The mice were treated for one year. There was no palpable tumor developed from either regular MCF-10A or from MCF-10A^{arom} cells.

In this study, we were unable to demonstrate tumorigenesis of MCF-10A^{arom} cells. Studies from others indicated that aromatase overexpression accelerated 7,12-dimethylbenz[*a*]anthracene-induced mammary tumor formation in aromatase transgenic mice (14). Our unpublished results from an in vivo model are also suggestive of the important role of estrogen metabolites in breast cancer initiation (15).

Key Research Accomplishments

- Establishment of aromatase expressing breast epithelial cell line
- Functional characterization of MCF-10A^{arom} cells
- Determination of estrogen metabolites and depurinating DNA adduct in MCF-10A^{arom} cells
- Anchorage independent growth of MCF-10A^{arom} cells
- Loss of heterozygosity assay
- in vivo tumorigenesis of MCF-10A^{arom} cells

Reportable Outcomes

1. "Tetracycline-controlled expression of aromatase" was submitted to *Era of Hope Department of Defense Breast Cancer Research Program Meeting,* 2002, Orlando, FL.
2. "Stable Expression of Aromatase in MCF-10A Benign Breast Epithelial Cells for Carcinogenesis Study" was submitted to *the 85th annual meeting of the Endocrine Society* for poster presentation in June 2003, Philadelphia, PA.
3. "Investigation of the carcinogenic effect of estradiol in the in vitro and in vivo models without estrogen receptors" was submitted to *the International Conference of Aromatase 2004* for poster presentation in September 2004, Edinburgh, United Kingdom.

Conclusions

MCF-10A^{arom} cells have all enzymes that catalyze formation of catechoestrogen and methoxyestrogen. Expression of aromatase increases in situ estrogen synthesis and its genotoxic products, which can be blocked by aromatase inhibitor, letrozole. Overexpression of aromatase in MCF-10A cells increases anchorage-independent growth in soft agar. MCF-10A^{arom} cells did not form palpable tumor in nude mice suggesting that multiple factors are required for initiation of breast cancer.

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Appendices

1. Abstract submitted to Era of Hope Department of Defense Breast Cancer Research Program Meeting, 2002.
2. Abstract submitted to the 85th annual meeting of the Endocrine Society, 2003.
3. Abstract submitted to the International Conference of Aromatase 2004.

APPENDIX 1

TETRACYCLINE-CONTROLLED EXPRESSION OF AROMATASE

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The relevance of estrogen to established breast cancer is well documented. Substantial correlative data suggest that estrogens cause breast cancer in women. The commonly held mechanism whereby estrogen causes cancer is that estrogen increases the rate of cell division and spontaneous replication errors while reducing time for DNA repair. However, a body of emerging data suggests another mechanism whereby estrogen is metabolized to genotoxic products that directly initiate mutations.

Conversion of androgen substrates to estrogens is catalyzed by aromatase, the rate-limiting enzyme in the estrogen synthesis pathway. While the circulating estrogen concentrations are low in postmenopausal women, estrogen levels in cancerous breast tissues are comparable to those found in breast cancers of premenopausal women. Our prior studies demonstrated that in situ aromatization in the breast play a critical role in determination of tissue estradiol concentration and tumor growth. We hypothesized that overexpression of aromatase in the breast causes breast cancer via metabolite-mediated genotoxicity and estrogen receptor-mediated cell proliferation.

To test this hypothesis, we plan to establish a benign breast epithelial cell line stably expressing aromatase and to determine carcinogenic metabolites and tumorigenesis of the cells. The CLONTECH Tet-Off Gene expression system is used as an approach to express aromatase. Human aromatase cDNA was amplified by PCR, purified, and ligated to pTRE vector. The resultant vector, pTRE-Arom was verified by sequence analysis. Existing MCF-7TetOff cells were stably transfected with pTRE-Arom and selected with G418. Of 86 G418-resistant clones, 42 showed aromatase activities of wide range by tritiated water release assay. Increased conversion of [3H]androstenedione can be inhibited by letrozole, a specific aromatase inhibitor. Treatment with doxycycline (100 ng/ml) for 3-4 days significantly reduced the conversion of [3H]androstenedione suggesting that aromatase expression was turned off. These data indicate that pTRE-Arom construct expresses functional aromatase protein under the control of tetracycline and is ready for controlled expression of aromatase in benign breast epithelial cells.

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APPENDIX 2

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Title: Stable Expression of Aromatase in MCF-10A Benign Breast Epithelial Cells for Carcinogenesis Study

Wei Yue [1]*, Jiping Wang [1] and Yuebai Li [1]. [1] Internal Medicine, University of Virginia, Charlottesville, VA, 22903 .

The relevance of estrogen to established breast cancer is well documented. Substantial correlative data suggest that estrogens cause breast cancer in women. The commonly held mechanism whereby estrogen causes cancer is that estrogen increases the rate of cell division and spontaneous replication errors while reducing time for DNA repair. However, a body of emerging data suggests another mechanism whereby estrogen is metabolized to genotoxic products that directly initiate mutations. Conversion of androgen substrates to estrogens is catalyzed by aromatase, the rate-limiting enzyme in the estrogen synthesis pathway. Our prior studies demonstrated that in situ aromatization in the breast play a critical role in determination of tissue estradiol concentration and tumor growth. We hypothesized that overexpression of aromatase in the breast increases estrogen concentration and causes breast cancer via metabolite-mediated genotoxicity and estrogen receptor mediated cell proliferation. To test this hypothesis, we plan to establish a benign breast epithelial cell line stably expressing aromatase for evaluation of estrogen metabolism and tumorigenesis. We transfected MCF-10A cells with aromatase gene under the human β-actin promoter and selected positive clones with G418. Thirty G418-resistant clones were collected for evaluation of aromatase activity using [1β-^{3}H] androstenedione as a substrate. Aromatase activity increased with time of incubation with the substrate. Letrozole, a specific aromatase inhibitor, inhibited aromatase activity in a dose-dependent manner. Km and Vmax of expressed aromatase were determined in three clones with the values of 6-9 nM (Km) and 4-15 pmol/mg/h (Vmax), respectively. Aromatase activity was verified by incubation of MCF-10A^{arom} cells with radiolabeled substrate, [1,2,6,7 ^{3}H]testosterone and

quantitation of estradiol (E_2) and estrone (E_1) after thin-layer chromatography. About 8% radioactivity was recovered from E_2 and E_1 spots, which was dose-dependently reduced by letrozole. The majority of the radioactivity was found in the water phase after ether extraction, which was reduced by 90% with letrozole. In the presence of letrozole, more than 90% substrate was converted to androstenedione through 17β-hydroxysteroid dehydrogenase compared to 5% without letrozole. Aromatase activity in MCF-10A^{arom} cells is comparable to those in other aromatase transfected cell lines reported previously.

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Keyword 1: Aromatase
Keyword 2: Transfection
Keyword 3: Breast cancer

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APPENDIX 3

Investigation of the carcinogenic effect of estradiol in the in vitro and in vivo models without estrogen receptors

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Estrogen causes cancers in animals. Substantial correlative data suggest that estrogens cause breast cancer in women. A body of emerging data suggests that estrogen is metabolized to genotoxic products that damage DNA and initiate cancer. This mechanism together with receptor-mediated stimulation of cell proliferation may act in concert to induce breast cancer. To test the genotoxic metabolite hypothesis, two models were employed: MCF-10A benign breast epithelial cells expressing aromatase were used to determine production of estrogen metabolites and their blockade by the aromatase inhibitor, letrozole. ERα knockout mice expressing the Wnt-1 oncogene (ERKO/Wnt-1) were chosen as an in vivo model to determine tumorigenesis in mammary glands where there is no functional ERα expressed. Incubation of MCF-10A^{arom} cells with androstenedione (10 μM) for 24 h resulted in an increase in the levels of 4-hydroxyestrone as well as its detoxicated product, 4-OHE-NAcCys, which was blocked by letrozole. These results indicate that breast epithelial cells have the enzymes that are required to convert estrogen to the genotoxic products. Overexpression of aromatase enhances in situ production of estrogen and its genotoxic metabolites. The ERKO/Wnt-1 mice were ovariectomized at 15 days. E_2 was administrated through s.c. silastic capsules. Predicted serum concentrations of E_2 delivered by silastic implants were 80 and 240 pg/ml. By 11 months, tumor incidences were 50% in animals receiving high dose E_2 and 25% with lower dose. In the control group only 16% of animals developed tumors ($p<0.04$). By 18 months, 66% of animals receiving high dose E_2 developed mammary tumors. Our data provide the first direct evidence that estrogen may cause breast cancer through a non estrogen receptor mediated mechanism.

Submitted to the International Conference of Aromatase 2004, Edinburgh, UK